Pulmonary Immunity to *Pseudomonas aeruginosa* in Intestinally Immunized Rats: Roles of Alveolar Macrophages, Tumor Necrosis Factor Alpha, and Interleukin-1α

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The aims of this study were to assess the role played by alveolar macrophages, tumor necrosis factor alpha (TNF-α), and interleukin-1α (IL-1α) in pulmonary immunity against *Pseudomonas aeruginosa* in animals that have been immunized via the gut-associated lymphoid tissue. Following intra-Peyer's patch immunization and subsequent intratracheal challenge with live bacteria, significantly enhanced bacterial clearance from the lungs correlated with an increase in bronchoalveolar neutrophils, increased recruitment and phagocytic activity of alveolar macrophages, and accelerated production of TNF-α in the bronchoalveolar space, while levels of IL-1α remained low. Administration of recombinant TNF-α in physiological concentrations did not affect the proliferation of *P. aeruginosa* in vitro, but when given intratracheally to rats at the time of infection, recombinant TNF-α significantly increased bacterial clearance from the lungs. In these animals, phagocytic activity of bronchoalveolar neutrophils was enhanced, while the recruitment of alveolar macrophages and neutrophils remained unchanged. In acutely infected nonimmune animals, bronchoalveolar concentrations of soluble IL-1α and TNF-α increased until the time of death. Levels of prostaglandin E2 and thromboxane B2 were similar in each experimental group. These results indicate that infection in immune animals enhanced both recruitment and phagocytic activity of alveolar macrophages as well as induced an accelerated production of TNF-α. In immune challenged animals, this cytokine enhanced the phagocytic activity of neutrophils and improved bacterial clearance from the lung. Levels of soluble IL-1α and TNF-α in nonimmune rats increased consistently following infection until the time of death, thus implicating these cytokines in the pathogenesis of acute *P. aeruginosa* pneumonia.

Infection with *Pseudomonas aeruginosa* is the major cause of morbidity and mortality in patients with cystic fibrosis (14). A number of studies have attempted to develop a vaccine against *P. aeruginosa* (reviewed in reference 3), but protective immunity and prevention of colonization in the lung have not been achieved. Moreover, the effector mechanisms of immunity against *P. aeruginosa* remain poorly understood. Our laboratory has developed an animal model in which intestinal immunization of rats with killed *P. aeruginosa* protects against subsequent pulmonary infection and significantly enhances survival (7). In this model, an enhancement of both neutrophil recruitment and activity was shown to be a central effector mechanism of protective immunity following intestinal immunization (5). Thus, cellular events appear to play a critical role in host defense against *P. aeruginosa*, an observation which is consistent with the findings of other studies (18, 20, 25, 27, 36). However, the mediators responsible for the activation of neutrophils in the immune lung remained undefined. In the airways, alveolar macrophages are pivotal effector cells of protective immunity because of their phagocytic activity, their role as an antigen-presenting cell, and their release of numerous products such as proteases, oxidizing agents, complement components, eicosanoids, and cytokines (1, 15, 27). Interleukin-1 (IL-1) and tumor necrosis factor (TNF), two proinflammatory cytokines mainly produced by macrophages, exist as two distinct polypeptides (IL-1α and IL-1β and TNF-α and TNF-β) (9). IL-1α and TNF-α share a wide range of biological activities, including the activation of neutrophils (9, 21, 29, 30, 33). In addition, it has been suggested that TNF-α on its own is lethal to *Pneumocystis carinii* (24) and capable of binding to gram-negative bacteria and altering their virulence properties (17). Other reports have shown that intramuscular or intraperitoneal administration of recombinant IL-1α or recombinant IL-1β, respectively, protected mice from lethal systemic *P. aeruginosa* infections (22, 37). In keeping with our efforts to further define the effector mechanisms of pulmonary immunity against *P. aeruginosa*, the aim of this study was to assess whether protective immunity in the lung following intestinal immunization was associated with an enhanced recruitment and/or activity of alveolar macrophages, which in turn may increase neutrophil activation via an increased production of TNF-α and/or IL-1α. The results indicate that (i) following a challenge infection, recruitment and early phagocytic activity of alveolar macrophages are significantly increased in animals that had been previously immunized, (ii) bronchoalveolar production of TNF-α but not IL-1α is accelerated in these rats, (iii) TNF-α on its own does not inhibit proliferation of *P. aeruginosa* in vitro, and (iv) intratracheal inoculation of recombinant TNF-α (rTNF-α) at the time of infection improves bacterial clearance by enhancing the phagocytic activity of bronchoalveolar neutrophils.
MATERIALS AND METHODS

Animal model. Male, specific-pathogen-free DA rats, 8 to 10 weeks old and weighing 180 to 280 g (mean $\bar{x}$ ± standard error of the mean [SEM], 222 ± 3 g), were used throughout this study as described previously (5, 7). Animals were fed commercial rodent pellets, given water ad libitum, and maintained under microisolator hoods in a specific-pathogen-free environment with 12-h-light–12-h-dark photoperiods until the start of the experimental protocol. Maintenance of animals and experimental procedures were carried out in accordance with the guidelines of the Ethics Committee of the University of Newcastle (Newcastle, New South Wales, Australia).

Bacteria. A mucoid P. aeruginosa isolate, serotype II, phage type 21/44/109/119X/1214 (Central Public Health Laboratory, London, United Kingdom), originally isolated from a patient with cystic fibrosis, was used and prepared in all experiments as described previously (5, 7). Briefly, P. aeruginosa was grown on chocolate agar plates overnight at 37°C, harvested, suspended, and washed in sterile phosphate-buffered saline (pH 7.3; 0.15 M NaCl; PBS), quantitated, and checked for purity by being replated for single-colony growth on chocolate agar. For immunization, bacteria were killed by exposure to 1% paraformaldehyde for 2 h and washed three times in sterile PBS.

Immunization procedures. Rats were immunized intra-Peyer’s patch as described previously (5, 7). Other studies have established that intra-Peyer’s-patch immunization mimics intestinal immunization and specifically induces gut-derived immune mechanisms, unlike intraperitoneal immunization which primes both mucosal and systemic immunity (11). Killed P. aeruginosa cells were suspended in sterile PBS to a concentration of $10^9$ CFU/ml as determined by photometry ($A_{405}$) and emulsified with an equal volume of incomplete Freund’s adjuvant. Following intravenous anesthesia of the rats with 3.6% chloral hydrate, 1 to 2 $\mu$l of the preparation was injected suberosally into each Peyer’s patch of the rats (designated immune animals). A second group of sham-treated animals (nonimmune animals) was inoculated with incomplete Freund’s adjuvant-PBS only. A third group of animals (control rats) was not immunized.

Challenge and bacterial clearance. Fourteen days postimmunization, nonimmune and immune rats were challenged intratracheally via a catheter (20 Ga) with 5 ml of PBS containing 5 x $10^8$ CFU of live autologous P. aeruginosa harvested from an overnight culture on chocolate agar. Control animals received PBS only. Rats were killed with an intraperitoneal overdose of pentobarbitone sodium (Nembutal; Boehringer Ingelheim, Artarmon, New South Wales, Australia) at 30 min, 4 h, 12 h, 24 h, and 7 days after intratracheal inoculation. After challenge, blood samples were collected by cardiac puncture, and bronchial washings were obtained by five sequential lavages with 2 ml of sterile PBS. Serum aliquots were frozen and kept at −70°C. Viable P. aeruginosa bacteria recovered in the bronchoalveolar lavage fluids (BAL) were counted by serial dilution and culture on nutrient agar for 18 h at 37°C. The remainder of each lavage sample was centrifuged for 10 min at 250 × g and 5°C. Aliquots from the supernatant were frozen and maintained at −70°C until further study. Bacterial recovery was expressed as log$_{10}$ CFU, and bacterial clearance in the immune animals was expressed as the percentage of cleared bacteria compared with the number of microorganisms recovered from nonimmune rats. Previous studies (2, 7) of pulmonary infections with P. aeruginosa or other microorganisms have clearly demonstrated that bacterial recovery from BAL was a reliable parameter which correlates with total bacterial clearance from the lungs.

Alveolar macrophage counts and phagocytic indices. Total bronchoalveolar cell counts were determined with a hemocytometer. In addition, 100 $\mu$l of each fresh BAL was spun for 10 min at 20 × g onto a microscope slide with a cytospin (Shandon, Inc., Pittsburgh, Pa.), and cytocentrifuged specimens were fixed and stained in Diff Quick (Veterinary Medical and Surgical Supply Pty, Ltd., Maryville, Australia) to allow the calculation of percentages of alveolar macrophages. Total numbers of alveolar macrophages were obtained by multiplying these percentages by the total number of bronchoalveolar cells. Total numbers of neutrophils were counted in a similar fashion. Cytospin slides were washed to remove free extracellular bacteria not associated with phagocytes, and the preparations were further evaluated under light microscopy for the determination of two phagocytic indices, i.e., (i) the percentage of alveolar macrophages that had phagocytosed 1 to 10 bacteria and (ii) the percentage of alveolar macrophages that phagocytosed more than 10 bacteria, as described previously (5).

Isolation and purification of alveolar macrophages. For the isolation of alveolar macrophages, BAL were centrifuged for 10 min at 250 × g and 5°C. The pellet was then resuspended in 1 ml of sterile PBS containing 0.1% (wt/vol) bovine serum albumin (BSA; Sigma-Aldrich, Bioscientific Ltd., Sydney, Australia). The cells in PBS-BSA were layered over a gradient of 20 and 60% Percoll (Pharmacia, Uppsala, Sweden) and centrifuged for 30 min, at 6,500 rpm, and 10°C. Alveolar macrophages were harvested from the 20 to 60% interface, washed in sterile PBS-BSA, centrifuged at 250 × g for 1 min, and suspended in 1 ml of sterile PBS. Cells were evaluated for viability by exclusion of trypan blue (0.1%), and the preparation was assessed microscopically for purity with a cytospin preparation as described above. Throughout the study, alveolar macrophage populations obtained in this fashion were >99% pure and >90% viable.

Electron microscopy. Phagocytosis of bacteria by alveolar macrophages was verified by use of transmission electron microscopy. Cells purified from immune animals after 30 min of bacterial challenge were centrifuged for 10 min at 250 × g and 5°C, fixed overnight at 5°C in Karnovsky’s fixative (2.5% glutaraldehyde, 2% formaldehyde), postfixed for 2 h at 4°C with 0.5% osmium tetroxide, dehydrated in ethanol, and embedded in Spurr’s resin (Spurr Resin). Sections (80 nm) were stained with saturated uranyl acetate in 50% ethanol and 0.04% lead citrate. Micrographs were obtained with a Philips CM10 transmission electron microscope at an acceleration voltage of 80 kV.

TNF-α and IL-1α assays. Levels of soluble IL-1α and TNF-α were measured in nondiluted BAL samples and diluted (1:2) serum specimens. The TNF-α concentration was determined with a mouse TNF-α enzyme-linked immunosorbent assay (ELISA) kit as described in the manufacturer’s instructions (Genzyme, Cambridge, Mass.). It has been previously established that murine TNF-α and rat TNF-α are cross-reactive and that this mouse ELISA kit could be used to detect rat TNF-α (6). Briefly, this ELISA is a solid-phase enzyme immunoassay employing the multiple-antibody sandwich principle in which TNF-α present in the samples is captured by a hamster monoclonal antibody for murine TNF-α previously adsorbed onto the wells. Following subsequent incubations with a goat polyclonal anti-murine TNF antibody, and with a horseradish peroxidase-conjugated donkey anti-goat immunoglobulin, the peroxidase enzyme reacts with the peroxide substrate and the chromogen o-phenylenediamine dihydrochloride added to the wells. The intensity of the color reaction, which is proportional to the amount of TNF-α from the sample, was read at $A_{492}$.
TABLE 1. Bacterial recoveries (CFU) and total alveolar macrophage and neutrophil counts in BAL from nonimmune and immune rats challenged for 0.5 and 4 h with live P. aeruginosa

<table>
<thead>
<tr>
<th>Challenge time and rat group</th>
<th>Bacteria (Log₁₀ CFU (% clearance)*</th>
<th>Alveolar macrophages (Count (10⁵)⁺</th>
<th>Neutrophils (Count (10⁵)⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 h</td>
<td>Nonimmune (n = 6-19)</td>
<td>7.63 ± 0.05</td>
<td>3.66 ± 0.44</td>
</tr>
<tr>
<td></td>
<td>Immune (n = 6-18)</td>
<td>7.11 ± 0.10* (69.8)</td>
<td>4.44 ± 0.50</td>
</tr>
<tr>
<td>4 h</td>
<td>Nonimmune (n = 4-12)</td>
<td>7.73 ± 0.08</td>
<td>1.81 ± 0.27</td>
</tr>
<tr>
<td></td>
<td>Immune (n = 6-13)</td>
<td>6.07 ± 0.09* (97.8)</td>
<td>23.78 ± 8.16*</td>
</tr>
</tbody>
</table>

* Values are x ± SEMs. * P < 0.05, nonimmune compared with immune values.

The assay was set up for a detection limit of 50 pg of TNF-α per ml. This ELISA kit has no detectable cross-reactivity with other cytokines (IL-1α, IL-2, IL-3, granulocyte-macrophage colony-stimulating factor and gamma interferon) (Genzyme).

Soluble IL-1α was measured with a competitive radioimmunoassay test kit which specifically quantitates rat IL-1α (Cytokine Sciences, Inc., Boston, Mass.). After incubating the primary rabbit anti-rat IL-1α antibody in the presence of IL-1 from the samples, 125I-radiolabelled recombinant rat IL-1α is added to the tubes as a tracer. Both the primary antibody and the tracer are used in limiting quantities. Hence, the amount of tracer that can bind to the primary antibody is inversely proportional to the amount of free IL-1 in the sample. A secondary antibody, sheep anti-rabbit immunoglobulin G, is then used to immunoprecipitate IL-1α–primary antibody complexes. Bound tracer is separated from unbound tracer by centrifugation, and the immunoprecipitate is counted directly with a gamma scintillation counter. The detection limit for this assay was 100 pg/ml of sample.

Effect of TNF-α on bacterial proliferation in vitro. In vitro dose response curves of bacterial proliferation versus physiological concentrations of TNF-α (covering the range of the levels measured in the in vivo experiments) were determined. Live P. aeruginosa bacteria (5 × 10⁸) in 100 μl of PBS were added to 5 ml of nutrient broth (0.8% peptone, 0.64% NaCl in dH₂O [pH 7.5]) prewarmed at 37°C. Increasing concentrations (0 [PBS alone], 0.28, 0.83, 2.5, 5, and 10 ng) of recombinant murine TNF-α (Genzyme) were added immediately to the preparations. Following incubation for 4 h at 37°C in a shaking incubator (200 rpm), viable bacteria in each tube were quantitated by serial dilution and culture on nutrient agar for 18 h at 37°C as described above. Bacterial proliferation factors were obtained from the ratio of CFU in each preparation at 4 h to the initial CFU at the time of inoculation.

Effects of TNF-α in vivo. Another set of experiments assessed the effects in vivo of rTNF-α on bacterial proliferation, neutrophil and macrophage recruitment, and phagocytic indices. Physiological concentrations (1, 4, and 10 ng) of murine rTNF-α (Genzyme), which is cross-reactive with rat TNF-α (6), were added to the intratracheal bolus of 5 × 10⁸ live P. aeruginosa bacteria as described above and given to nonimmune animals. The results were compared with those obtained from one group of nonimmune rats that received P. aeruginosa in PBS only. Animals were killed 3 h postchallenge, and the BAL was collected for assessment of bacterial recovery, macrophage and neutrophil counts, and calculation of macrophage and neutrophil phagocytic indices, as described above.

PGE₂ and TXB₂ assays. Prostaglandin E₂ (PGE₂) and thromboxane B₂ (TXB₂) synthesis was measured in nondiluted and diluted sera and BAL samples (serum, 1:200; BAL, 1:10) with a competitive enzyme immunometric assay kit as described in the instructions provided by the manufacturer (Cayman Chemical Company, Sapphire Bioscience, Alexandria, New South Wales, Australia). Briefly, the immunoassay for each eicosanoid was based on the competition between free PGE₂ or TXB₂ in the samples and acetylcholinesterase-linked PGE₂ or TXB₂ tracer for limited specific rabbit antiserum-binding sites. Thus, the amount of tracer that is able to bind the rabbit antiserum will be inversely proportional to the concentration of free eicosanoid. The eicosanoid-antiserum complexes bind to a mouse monoclonal anti-rabbit antibody previously adsorbed onto the well, and after the addition of Ellman’s reagent, a color reaction which can be measured spectrophotometrically (A₄₁₅) develops. The amount of free PGE₂ or TXB₂ is inversely proportional to the intensity of the color reaction. The PGE₂ immunoassay is 100% specific for PGE₂, 9.2% for 15-keto PGE₂, 5% for PGE₃, and <0.2% for TXB₂ and other prostaglandins. The TXB₂ immunoassay is 100% specific for TXB₂, 8.2% for 2,3-dinor TXB₂, 0.2% for 11-dehydro-TXB₂, and <0.01% for PGE₂, PGE₃, and leukotriene B₄ (LTB₄).

Statistical analysis. All results were expressed as x ± SEM compared by one-way analysis of variance and then by Tukey’s test for multiple-comparison analysis when applicable. When results were expressed as percentages, all values under 50% were arcsine transformed prior to statistical comparison and were transformed back to percentages for the expression of x ± SEM. Levels of P of <0.05 were considered significant.

RESULTS

Bacterial clearance and macrophage counts. Bacterial recoveries and numbers of macrophages and neutrophils counted in the BAL are given in Table 1. Intra-Peyer’s-patch immunization with killed bacteria resulted in significantly enhanced bacterial clearance from the lung after subsequent pulmonary challenge with live P. aeruginosa. Compared with nonimmunized rats, immune animals had cleared 69.8% bacteria from their lungs after 30 min and 97.8% after 4 h. This rate of clearance was associated with a significantly enhanced recruitment of alveolar macrophages 4 h after challenge, while neutrophil recruitment was significantly increased at 30 min and 4 h. The numbers of alveolar macrophages in immune and nonimmune animals were not different prior to challenge (data not shown).

Phagocytic indices. Examination of cytospin preparations under light microscopy allowed easy identification of phagocytes that had ingested P. aeruginosa (Fig. 1). Intracellular localization of the phagocyted bacteria was confirmed with transmission electron microscopy (Fig. 1). After 30 min of live
bacterial challenge, alveolar macrophages that had phagocy-
tosed more than 10 bacteria were found in significantly \( (P < 0.01) \) greater concentrations in the BAL from immune animals than in those of nonimmune animals (Fig. 2). However, at both 30 min and 4 h, there was no difference between the percentages of macrophages that had ingested 1 to 10 microorganisms. The overall phagocytic activity of alveolar macrophages de-
creased significantly over time, and after 4 h of challenge, macrophages that had phagocytosed more than 10 bacteria were no longer detected in either experimental group (Fig. 2).

**TNF-\( \alpha \) and IL-1\( \alpha \) measurements.** Levels of soluble TNF-\( \alpha \) and IL-1 measured in the BAL are illustrated in Fig. 3 and 4, respectively. TNF-\( \alpha \) was found in BAL after challenge with live *P. aeruginosa* but not in control animals given PBS. Concentrations of pulmonary TNF in nonimmune and immune ani-
imals were not different 30 min after intratracheal challenge, but levels rose more rapidly in the lungs of immune animals thereafter (at 4 h, nonimmune group concentration = 1.63 \( \pm \) 0.21 ng per lung \( [n = 8] \); immune group concentration = 4.35 \( \pm \) 0.54 ng per lung \( [n = 11] \), \( P < 0.001 \) compared with nonimmune group). While TNF continued to increase in the nonimmune rat lungs after 4 h, a sharp decrease of TNF levels was measured in the immune samples \( (P < 0.01 \) compared with nonimmune samples at 12 h) (Fig. 3). At day 7, TNF was undetectable in BAL from immune animals. TNF-\( \alpha \) was not detected in serum samples from control, nonimmune, and immune animals 30 min and 4 h after intratracheal inoculation (data not shown).

In BAL of immune animals, levels of soluble IL-1\( \alpha \) re-
mained low throughout the time of study (Fig. 4). In contrast, in BAL of nonimmune rats, concentrations of the free cytokine rose rapidly in response to the bacterial infection, until the
were bacteria significantly changed in animals of all ages in a dose-dependent fashion (Table 2). At this time, however, phagocytic indices of neutrophils were significantly increased in a dose-dependent fashion (Fig. 6). Only very low percentages (<5%) of alveolar macrophages contained phagocytosed bacteria in either experimental group (data not shown).

**PGE\(_2\) and TXB\(_2\) synthesis.** PGE\(_2\) syntheses were not significantly different between immune and nonimmune animals 0.5 h after challenge (serum concentrations in nonimmune and immune animals were 2.4 ± 0.5 and 3.5 ± 1.0 ng/ml, respectively; BAL concentrations in nonimmune and immune rats were 1.7 ± 0.2 and 1.5 ± 0.1 ng/ml, respectively) or 4 h after challenge (serum concentrations in nonimmune and immune rats were 10.0 ± 3.9 and 12.0 ± 3.3 ng/ml, respectively; BAL concentrations in nonimmune and immune rats were 1.4 ± 0.2 and 1.5 ± 0.1 ng/ml, respectively [values are ± SEMs; \(n = 5\) to six animals]). Similarly, syntheses of TXB\(_2\) were not different between the two groups (data not shown).

**DISCUSSION**

Findings from this study demonstrate that alveolar macrophages contribute to pulmonary protection against *P. aeruginosa* in a host that had been previously immunized via the gut-associated lymphoid tissue. Enhanced bacterial clearance from the immune lung was associated with increased recruitment and phagocytic activity of alveolar macrophages coupled with accelerated production of TNF-\(\alpha\) in the bronchoalveolar space. Physiological concentrations of rTNF-\(\alpha\) did not affect the proliferation of *P. aeruginosa* in vitro. However, when given intratracheally at the time of infection, rTNF-\(\alpha\) improved bacterial clearance from the lungs, an effect that was associated with a dose-dependent increase of the phagocytic activity of bronchoalveolar neutrophils. Bronchoalveolar levels of soluble IL-1\(\alpha\) in immune animals remained low throughout the entire experimental time, and concentrations of PGE\(_2\) or TXB\(_2\) were not different between immune and nonimmune rats. Consistent with our previous observations (5, 7), these results confirm that intra-Peyer's-patch priming can protect the lung against infection with *P. aeruginosa*, and the present study suggests that alveolar macrophages and TNF-\(\alpha\) are implicated in the immune cascade leading to this protection.

Macrophages play a prominent role in pulmonary protection against acute bacterial infections. *P. aeruginosa* can be phagocytosed by macrophages in the presence or absence of serum...
nosa (5), critical observations have been increased in numbers of pulmonary immunity against phages. In immune cells via culture in media, none (solid bars) were not significantly different from those which had increased numbers (empty bars). Values are ±SEMs of results from four experiments in each group.

Moreover, in immunized mice, macrophages appear to be the final effector cells in protective pulmonary immunity against Listeria monocytogenes (36). In the present study, enhanced pulmonary clearance of P. aeruginosa in immune animals was associated with a significant increase in the recruitment and phagocytic activity of alveolar macrophages. In addition, the present report confirms that increased numbers and phagocytic activity of neutrophils are a critical component of pulmonary immunity against P. aeruginosa (5), as had been shown for Haemophilus influenzae (40). These observations indicate that intestinal immunization enhances the phagocytic capacity of both the macrophages and the neutrophils in the lung. The degree of specificity of this increased activity deserves to be investigated further.

The diverse functions of alveolar macrophages by far exceed the simple role of phagocytes. Among their other biological activities, they are capable of modulating the activity of other immune cells via numerous secretory products (1, 9, 27). Upon stimulation, two of the most prominent cytokines produced by the macrophages are TNF-α and IL-1. Recent studies have associated an increased production of IL-1 and TNF-α with pathogenesis and/or protective immunity in a number of pulmonary disorders, including allergic airway disease (41), chronic obstructive pulmonary disease (39), and infections with Mycobacterium tuberculosis (44), Candida albicans (39), or P. carinii (16). In addition, exposure to P. aeruginosa stimulates mononuclear leukocytes to release IL-1 and TNF-α in vitro (32). These observations unequivocally demonstrate that production of IL-1 and TNF-α in the lung constitutes a major component of the host response to acute pulmonary disease. Accordingly, in the present study, in acutely infected nonimmune rats, pulmonary levels of soluble TNF-α and IL-1α increased consistently until the time of death. Moreover, following immunization, production of TNF-α was significantly accelerated in the lung in response to a challenge with live P. aeruginosa, indicating that this cytokine may mediate the increased pulmonary protection observed in a vaccinated host. In contrast, bronchoalveolar levels of soluble IL-1α remained low for reasons which require further investigation. Recent studies

![Graph](http://iai.asm.org/)

**FIG. 5.** Proliferation ratio of P. aeruginosa in vitro after 4 h of culture in nutrient broth to which various amounts of rTNF-α had been added. Ratios calculated from preparations containing rTNF-α (empty bars) were not significantly different from those which had none (solid bars). Values are ±SEMs of results from four experiments in each group.

![Graph](http://iai.asm.org/)

**FIG. 6.** Percentages of bronchoalveolar neutrophils that have phagocytosed P. aeruginosa 3 h postchallenge in rats that were inoculated intratracheally with 10,000, 4,000, or 1,000 pg of rTNF-α or none. Values are ±SEMs of results with eight animals in each group. *, P < 0.01 compared with animals that received no TNF.

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**TABLE 2.** Bacterial recoveries (CFU) and cell counts in BAL from rats that received various doses of intratracheal rTNF-α or the vehicle alone at the time of infection

<table>
<thead>
<tr>
<th>rTNF-α doses (pg)</th>
<th>Log_{10} CFU of bacteria (% clearance)</th>
<th>Cell count (10³)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total leukocytes</td>
</tr>
<tr>
<td>0</td>
<td>8.36 ± 0.09</td>
<td>18.0 ± 3.3</td>
</tr>
<tr>
<td>1,000</td>
<td>8.24 ± 0.07 (25.0)</td>
<td>19.3 ± 9.7</td>
</tr>
<tr>
<td>4,000</td>
<td>7.96 ± 0.07* (60.3)</td>
<td>18.8 ± 1.5</td>
</tr>
<tr>
<td>10,000</td>
<td>8.29 ± 0.07 (15.9)</td>
<td>17.5 ± 1.4</td>
</tr>
</tbody>
</table>

* All animals were challenged for 3 h with live P. aeruginosa.

* Values are ±SEMs of results with eight animals in each experimental group.

* P < 0.01 compared with animals that received no rTNF-α.
have demonstrated that IL-4 and IL-10 increase the synthesis of IL-1 receptor antagonist and concurrently decrease that of IL-1 (8, 13, 38). Both IL-4 and IL-10 are produced by T cells, which are found in a higher state of activation in animals immunized against *P. aeruginosa* (10). Alternatively, as it has been established that LTB₄ is a potent enhancer of IL-1 production by lipopolysaccharide-stimulated monocytes (28), the significantly lower levels of LTB₄ measured in the bronchoalveolar space of immune animals from the present study (5) may also contribute to the lower concentration of IL-1α found in the same samples. Further investigations using this model will determine whether the controlled levels of IL-1α measured in the immune lung may in fact result from a higher T-cell-dependent IL-4 and/or IL-10 production and whether these changes correlate with inhibition of LTB₄ synthesis. Because IL-1 and TNF-α are potent inducers of inflammation, various methods to control their production and/or activity in situ are currently being developed in an attempt to prevent their deleterious side effects (8, 9). The study reported here shows for the first time that intestinal immunization may significantly reduce the long-term production of these cytokines at an inflammatory site. Following a live bacterial challenge, although the production of TNF-α was significantly enhanced in immune animals at 4 h, this peak was followed by a rapid drop by 12 h postchallenge. In contrast, in the lungs of nonimmune animals, levels of TNF-α consistently increased until the time of death. Moreover, levels of soluble IL-1α remained low in immune rat lungs throughout the time of study, while rising concentrations of the soluble cytokine were measured in the dying nonimmune animals. Interestingly, it has been reported that in cystic fibrosis patients colonized with *P. aeruginosa*, levels of endobronchial IL-1 and TNF-α are dramatically increased compared with control values from noninfected patients and that pulmonary inflammation correlates with cytokine concentration (34, 42). Taken together, these observations implicate both cytokines in the pathogenesis of acute *P. aeruginosa* pneumonia. The pattern of the cytokine response in immune animals strongly contrasted with that seen in nonimmune animals and correlated with improved pulmonary protection, indicating that accelerated TNF-α production and controlled levels of soluble IL-1α in the lung may contribute to a rational therapeutic strategy established for the development of an effective vaccine against *P. aeruginosa* pneumonia and possibly other pulmonary pathogens.

The role played by TNF-α in pulmonary immunity against *P. aeruginosa* was studied further. TNF-α alone had no significant effect on the proliferation of *P. aeruginosa* in vitro, which implies that this cytokine improves bacterial clearance from the lung indirectly via its immunomodulatory pathway rather than because of a direct bactericidal effect. The contrasting findings reported on the microbicidal effect of TNF-α on *P. carinii* in vitro (24) may represent a dose-dependent phenomenon since killing in the latter study could be observed only with TNF-α concentrations significantly higher than those used in the present experiments. In vitro, TNF-α can augment the phagocytic and cytotoxic activities of neutrophils (30). Gamma interferon and TNF-α also prime peritoneal macrophages for enhanced antibody-dependent cellular cytotoxicity (12), and these two cytokines potentiate the uptake and intracellular killing of opsonized *P. aeruginosa* by peritoneal macrophages (26). The latter observations are in keeping with the enhanced phagocytic indices of alveolar macrophages in vivo reported in the present investigation. That TNF-α can in part mediate the immune cascade discussed here was confirmed when the recombinant cytokine was administered intra-tracheally at the time of infection. Indeed, animals that received 4,000 pg of rTNF-α saw their bacterial clearance improve, an effect associated with a dose-dependent activation of the phagocytic activity of bronchoalveolar neutrophils. However, inoculation of rTNF-α did not affect the recruitment of neutrophils into the bronchoalveolar space in vivo. Because enhanced endobronchial neutrophilic chemotaxis and chemokinesis in immunized animals have been reported previously (5), other mediators, perhaps such as granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, and/or IL-8, must be involved in the immune events seen in the present system. Granulocyte colony-stimulating factor, for example, has been shown to suppress the growth of *P. aeruginosa* in the thigh muscles of experimentally infected mice (43). Implication of other factors is further suggested by the observation that bacterial clearance was not significantly increased by 10,000 pg of endobronchial rTNF-α, despite the concurrent enhancement of phagocytic activity in bronchoalveolar neutrophils. Further investigation using this animal model will likely lead to the identification of other mediators of pulmonary immunity in intestinally vaccinated animals. In addition, such studies will help determine whether TNF-α is strictly necessary for this cascade of events to be initiated, or, alternatively, whether these other immune mediators may trigger successful protective immunity in the absence of TNF-α.

*P. aeruginosa* exhibits numerous immunooevasive activities (4), and one study in vitro demonstrated that elastase and alkaline protease released by the bacteria could inactivate human gamma interferon and, to a lesser extent, TNF-α (23), effects which have the potential of impairing cellular immunity. However, the present report and previous findings (5) show that pulmonary protection in immune animals correlates with enhanced recruitment and activity of alveolar macrophages and neutrophils and that these cellular events are associated with an accelerated production of TNF-α in the lung. The results imply that at least part of the immunooevasive activities displayed by *P. aeruginosa* in vitro can be overcome in vivo and that cell-mediated protection remains effective. Preliminary findings from studies using the same animal model have implicated T cells in this mechanism of protection (10), and other investigators have reported an interaction of T cells and peritoneal macrophages in vitro during cell-mediated immunity against *P. aeruginosa* (18). Similarly, our recent report showed that the best defense against intracellular infections with *M. bovis* implicates a CD4⁺ T-cell-mediated macrophage activation which involves gamma interferon and TNF-α and requires direct physical contact between effector lymphocytes and macrophages (35). Since physical interactions between endobronchial lymphocytes and macrophages were observed in the present system (Fig. 1A), the role played by direct cell contact of CD4⁺ T cells with phagocytes in anti-*P. aeruginosa* pulmonary immunity deserves to be investigated.

In previous studies using the same experimental system as the one described here, findings had indicated that upon bacterial challenge, levels of LTB₄ in a 5⁻lipoxigenase metabolite, were significantly lower in the lungs of immune animals than in those of nonimmune animals (5). As both IL-1 and TNF-α can induce PGE₂ synthesis in a variety of tissues (9) and because of the well-known immunosuppressive activities of PGE₂, it became particularly relevant to evaluate the involvement of cyclooxygenase metabolites in the present experimental model. PGE₂ and TXB₂ levels did not differ between any groups, either 30 min or 4 h after infection, thus implying that these compounds are not implicated in the early postchallenge phase of pulmonary immunomodulation in immune animals challenged with *P. aeruginosa*.

In summary, adding to the well-described role of alveolar...
macrophages as protective scavengers and antigen-presenting cells during an acute infection with *P. aeruginosa*, findings from the present study unequivocally demonstrate that when a host has been intestinally vaccinated, these phagocytes play a pivotal role in mediating protective pulmonary immunity via their enhanced recruitment and phagocytic activity early in the challenge infection. In addition, intestinal immunization primes the lung for accelerated production of TNF-α, which seemingly enhances phagocytosis of bacteria by neutrophils, thus further improving bacterial clearance from the lung. We postulate that following intestinal immunization with *P. aeruginosa*, pulmonary protection against a subsequent infection is achieved regardless of the immunoevasive activities of the bacteria and that this protective immunity is mediated by activated T cells and their cytokines, which stimulate alveolar macrophages and neutrophils, hence initiating a self-amplifying immunological reaction. Current studies are in the process of evaluating the implication of colony-stimulating factors in this system. Such investigations will help establish a rational basis for the development of novel immunotherapies in the treatment of pulmonary infections with *P. aeruginosa*, a research area of foremost priority in the clinical management of patients with cystic fibrosis (3).

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